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Downregulation of Metallothionein 1F, a putative oncosuppressor, by loss of heterozygosity in colon cancer tissue

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ABSTRACT

Purpose: Downregulation of *metallothionein* (*MT*) genes has been reported in several tumors with discrepant results. This study is to investigate molecular mechanism of MT gene regulation in colon cancer which is characterized by tumor suppressor gene alterations.
Experimental design: Integral analysis of microarray data with loss of heterozygosity (LOH) information was employed. Quantitative real-time PCR and immunohistochemistry were used to validate MT isoform expression in colon cancer tissues and cell lines. The effects of MT1F expression on RKO cell survival and tumorigenesis was analyzed. Bisulphite sequencing PCR (BSP) and methylation-specific PCR were employed to detect the methylation status of the *MT1F* gene in colon cancer tissues and cell lines. DNA sequencing was used to examine the LOH at the MT1F locus.
Results: MT1F, *MT1G*, *MT1X*, and *MT2A* gene expression was significantly downregulated in colon cancer tissue ($p<0.05$). Exogenous MT1F expression increased RKO cell apoptosis and inhibited RKO cell migration, invasion and adhesion as well as in vivo tumorigenicity. Downregulation of *MT1F gene* in majority of human colon tumor tissues is mainly through mechanism by loss of heterozygosity ($p=0.001$) while CpG island methylation of *MT1F gene* promoter region was only observed in poorly differentiated, MSI-positive RKO and LoVo colon cancer cell lines.
Conclusions: MT1F is a putative tumor suppressor gene in colon carcinogenesis that is downregulated mainly by LOH in colon cancer tissue. Further studies are required to elucidate a possible role for MT1F downregulation in colon cancer initiation and/or progression.

1. Introduction

Despite increased detection in recent years, colon cancer remains a major cause of mortality, representing the third most fatal malignancy in the world [1,2]. Colon cancer arises as a result of the accumulation of genetic and epigenetic changes that transform normal colon epithelium into adenocarcinoma [3]. Chromosomal instability, which is characterized by abnormal chromosome number and loss of heterozygosity (LOH), appears to play a major role in colon cancer pathogenesis [4], resulting in altered tumor suppressor gene (TSG) and oncogene expression and subsequent tumor progression [5]. Genome-wide LOH scanning and refined mapping of candidate loci revealed nine loci as having high LOH frequencies in sporadic colon cancer [6]. However, the contribution of gene-specific alterations in colon cancer progression remains to be fully elucidated. In particular, the role of TSGs has yet to be explored.

Xiao et al. [7] reported that 33% of Chinese colon cancer patients had a deletion hotspot of chromosome 16q11.2–21. Further refined mapping revealed that 16q13.1, which contains *metallothionein* (*MT*) genes, had a high LOH frequency. MTs are low molecular weight (6–7 kDa) cysteine-rich proteins [8]. Human MT proteins are encoded by a family of genes containing two ubiquitous isoforms, MT1 and MT2, and two tissue-specific isoforms, MT3 and MT4; MT3 and MT4 expression is restricted to the brain and squamous epithelia of the skin and tongue, respectively [9–11].

Discrepant results regarding MT protein expression in human cancers have been reported. MT expression was upregulated in breast, lung, kidney and pancreatic cancers, and it was often correlated

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with treatment resistance and poor prognosis [9]. However, in hepatocellular, gastric, central nervous system, and thyroid cancers, MT expression is downregulated, inversely correlated, or unrelated to mortality [8]. In some studies, MT was repressed in primary colon cancer and metastatic lesions, and its expression was inversely correlated with tumor development [12–14]. Other studies reported that MT expression was associated with tumor infiltration, Dukes' stage, and poor survival [15–17]. Furthermore, microarray data revealed that the *MT1F* gene was downregulated in normal-adenoma–carcinoma sequence of colon tissues [18]. In colon cancer cell lines, MT mRNA isoform expression was basally repressed [19]. Differential regulation of *MT* gene isoforms in various tumors may account for these discrepant results [8].

The present study aimed to further explore MT isoform expression in colon cancer. Specifically, the expression profile of *MT* genes was determined in colon cancer tissues as well as colon cancer cell lines using integral analysis of LOH loci and previously obtained microarray data [20] that was subsequently validated by quantitative real-time polymerase chain reaction (QRT-PCR). Because the *MT1F* gene was downregulated in normal-adenoma–carcinoma sequence of colon tissues [18], this study specifically analyzed its protein expression using immunohistochemistry and tissue microarrays (TMAs). The influence of MT1F expression on RKO colon cancer cell growth, migration, invasion and tumorigenicity was also analyzed.

2. Materials and methods

2.1. Clinical samples and cell lines

This study was approved by the Institutional Review Board of the Shanghai First People's Hospital. Tissue samples were collected from 40 colon cancer patients (21 male and 19 female; median age of 67.5 y, range 48–87 y), who had undergone tumor resection without receiving preoperative therapy, at the General Surgery Department. The clinicopathologic parameters of each patient were shown in Table S1. The fresh cancerous mucosa and adjacent normal mucosa (10 cm distant from the original tumor site) were immediately frozen in liquid nitrogen and stored at −80 °C until DNA/RNA extraction for quantitative real time PCR (QRT-PCR). Tumor stage classification was carried out as previously described [21]. Tissue samples for Affymetrix HG U133 Plus 2.0 GeneChip experiments or TMA construction were harvested as previously described [20,22].

Six human colon cancer cell lines, RKO, HCT116, HT29, SW620, CW-2 and LoVo cells, were obtained from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Type Culture Collection of the Chinese Academy of Sciences (Shanghai, PR China). The cells were cultured under conditions suggested by the vendors.

2.2. Laser capture microdissection (LCM) microarray analysis

Tissue sample harvest, LCM, RNA extraction, T7 amplification, and microarray hybridization were undertaken as described previously [20]. Microarray data was analyzed using GeneChip Operating Software (Affymetrix, Santa Clara, CA, USA). Expression values and relative expression values (Signal Log Ratio, Change) were calculated according to Affymetrix Statistical Algorithms Descriptions. The Gene Set Enrichment Analysis was performed using Onto-Express at the website http://vortex.cs.wayne.edu/Projects.html. Genes with a signal log ratio ≤ -1 were considered downregulated. Finally, the integration of downregulated genes with LOH loci was performed to identify new TSGs.

2.3. QRT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen) after which cDNA was produced from 1 μg RNA using an A3500 reverse-transcription PCR System (Promega Corporation, Madison, WI, USA). An aliquot of diluted cDNA equivalent to 10 ng total RNA was used for QRT-PCR analysis employing the ABI Prism 7500 system (Applied Biosystems Inc., Foster City, CA, USA) and primers specific for each MT isoform [23]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control; the primers were sense 5′-GTCCACCACCCTGTTGCTGTA-3′ and antisense 5′-CTTCAACAGCGACACCCACTC-3′. QRT-PCR reactions were repeated in triplicate using a 2×SYBR Green PCR Master Mix (Applied Biosystems) and previously reported amplification conditions [24]. The fold change ($2^{-\Delta\Delta Ct}$) in each tumor was compared with paired non-tumorous tissue.

2.4. TMA construction and immunohistochemistry

TMA construction was undertaken as previously reported [22]. Briefly, paraffin-embedded TMA sections were dewaxed and rehydrated before antigen retrieval was carried out. Immunolabeling was carried out using a mouse anti-MT monoclonal antibody (ab12228, Abcam, Cambridge, United Kingdom), which is specific for both MT1 and MT2 isoforms, diluted 1:200. Then, the sections were incubated with an anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP; DAKO Envision System, DAKO, Carpinteria, CA, USA), and then 3,3′-diaminobenzidine (DAB). The pattern of MT staining was also characterized as both cytoplasmic and nuclear distribution as previously described [13,14,16]. MT expression was evaluated by two pathologists independently, according to the intensity of the color reaction and the number of positive cells as described by Dziegiel et al. [16].

2.5. Transfection of MT1F in colon cancer cell lines

Gene-specific primers for cloning MT1F were designed (Table S2) and MT1F cDNA was amplified by PCR then subcloned into pEGFP-C1 (Invitrogen), generating pEGFPC1-MT1F, which was verified by sequencing. RKO cells (5×10^5 per well) were seeded in 6-well plates and cultured to 60–70% confluence, after which they were transiently transfected with pEGFPC1-MT1F or empty vector controls using Lipofectamine 2000 (Invitrogen). For stable transfectants, medium was replaced after 24 h with G418-containing medium (400 μg/mL; Gibco/Life Technologies, Gaithersburg, MD, USA). After 2 weeks, colonies with fluorescence were expanded. MT1F expression was confirmed using QRT-PCR or Western blot analysis using the anti-MT monoclonal antibody.

2.6. Western blot analysis

Proteins from 40 paired frozen colon tumor and adjacent normal tissue samples were extracted using Radio Immunoprecipitation Assay (RIPA) lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) with phenylmethanesulfonyl fluoride (17.4 μg/μL) and a protease inhibitor cocktail (0.89 μg/μL, Sigma-Aldrich). Equivalent amounts of protein were separated on SDS-polyacrylamide gels followed by transfer to polyvinylidene difluoride membranes, which were blocked in 5% fat-free milk with TBS-Tween-20 at room temperature for 1 h, followed by incubation with either the mouse anti-MT monoclonal antibody (1:500) or anti-β-actin monoclonal antibody (1:5000, Epitomics, California, USA) for 2 h at room temperature. Following incubation with a goat-anti-rabbit IgG (H+L) HRP-conjugated secondary antibody (1:5000, Santa Cruz or 1:1000, Promega, Wisconsin, USA), the blots were visualized using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, USA) and exposure to Syngene GBOX/iCHE gel imaging and analysis systems.

2.7. Cell growth and colony formation assay

Exponentially growing RKO, RKO-pEGFPC1, and RKO-pEGFPC1-MT1F cells were trypsinized and re-suspended in EMEM supplemented with 10% FBS, and then seeded onto 12-well plates (6×10^4 cells per well). The cells were incubated for 7 days, and the number of living cells in each well was determined daily using a CASY DT Cell Counter (Innovatis AG, Reutlingen, Germany). The experiments were independently repeated three times.

Colony formation was determined by preparing single cell suspension solutions and seeding 35-mm dishes with 500 cells. After cells were incubated for 12 days, the colonies were washed twice with PBS, fixed with pure methanol in −20 °C for 30 min, and stained with crystal violet for 10 min. Stained colonies were counted, and the plates were photographed. All experiments were performed in triplicate.

2.8. Migration, invasion, and adhesion assays

The migration assay was performed using 24-well Boyden chambers with polycarbonate membranes of 8 μm pore size (Corning Inc., New York, USA). For the invasion assay, the CytoSelect™ Cell Invasion Assay Kit (Cell Biolabs, Inc., San Diego, CA) was used according to the manufacturer's protocol. Cells were cultured for 12 h in serum-free medium. A cell suspension containing 5×10^5 cells/mL in serum-free media containing 0.1% bovine serum albumin (BSA) was prepared; 200 μL was added to the upper chamber and 500 μL media with 20% FBS was added to the lower chamber. After 48 h for the migration assay and 72 h for the invasion assay, the incubation was terminated, and the cells bound to the lower surface of the membrane were fixed with 95% ethanol and stained with crystal violet. The photographs were taken under high magnification with a microscope (Nikon, Tokyo, Japan).

Cell adhesion was determined using the CytoSelect™ 48-well Cell Adhesion Assay Kit (Cell Biolabs) following the recommended protocol. A cell suspension containing 0.1×10^6 cells/mL was prepared in serum-free media, and 150 μL was added to each well. After incubation at 37 °C for 90 min, each well was washed 4 times with 250 μL PBS and incubated with 200 μL crystal violet for 10 min at room temperature. After the cells were washed 4 times with 500 μL deionized water, the wells were air-dried for 30 min.

For quantitative analysis of each experiment, the crystal violet was dissolved in 200 μL extraction solution per well, and the absorbance was measured at 590 nm in a plate reader (Bio-Rad, Hercules, CA, USA). All experiments were done in triplicate.

2.9. Apoptosis and cell cycle analysis

Cell cycle progression and apoptosis were analyzed using the Annexin V-FITC Apoptosis Kit (BD Biosciences, Palo Alto, CA, USA). After a 48 h transfection, subconfluent cells underwent overnight serum starvation after which they were harvested, rinsed with PBS, and fixed in 70% ethanol for 1 h at 4 °C. Apoptosis rates were determined using a FACSCalibur flow cytometer (BD Biosciences). At least 1×10^4 cells were captured in each sample.

Total DNA was extracted from cells using an Apoptosis DNA Ladder Detection kit (KeyGen Biotech., Nanjing, China), according to the manufacturer's recommendation. DNA fragments were visualized under ultraviolet light in contrast to a DNA marker (DL2000™, TaKaRa Bio).

2.10. Tumorigenicity in nude mice

Animal studies were carried out in accordance with the guidelines established by the Shanghai Resource Center of Laboratory Animal, Chinese Academy of Science. Six 4- to 6-week old (18 to 20 g) BALB/c nu/nu mice per group were subcutaneously injected into the right flank with 5×10^6 cells in 0.25 mL PBS. The mice were sacrificed on day 21 after tumor implantation, and tumor weight and volume were quantified. A sliding caliper was used to measure the size of tumors, and the volume of tumors was calculated using the following formula: $\text{width}^2\times\text{length}\times0.5$.

2.10.1. Isolation and bisulfite treatment of genomic DNA and bisulfite sequencing

Genomic DNA was isolated from cell lines and tissue samples using the AxyPrep genomic DNA Miniprep kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's protocol. Cell lines were grown for 72 h in the presence or absence 10 μmol/L of 5-Aza-2′-deoxycytidine (5-Aza-dC), a known DNA methyltransferase inhibitor (Sigma-Aldrich), before they were harvested for DNA or RNA extraction. DNA (1 μg) underwent bisulfite conversion using the MethylCode Bisulfite Conversion Kit (Invitrogen). In brief, CT Conversion Reagent was added to the DNA sample and the following PCR reaction was undertaken: 98 °C for 10 min, 64 °C for 2.5 h, and 4 °C for up to 20 h. The DNA was purified in Binding Buffer and incubated in Desulphonation Buffer for 20 min at room temperature. After Wash Buffer precipitation, the acquired bisulfite-treated genomic DNA was dissolved in 10 μL Elution Buffer.

The methylation status of all colon cancer cell lines was analyzed by direct bisulfite sequencing of the MT1F promoter. The 5′ region of the *MT1F* gene was amplified using the following primers: sense, 5′-GGGATTTTAGGAAAGGTTTAGTTGT-3′ and antisense, 5′-CTCTCAAA ATCTTAACCAAAAAAAA-3′. The fragment covered 41 CpG sites and was amplified using Platinum Taq DNA polymerase High Fidelity (Invitrogen). PCR conditions were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 40 s; and a final extension of 10 min at 72 °C. The PCR products were gel-purified with AxyPrep DNA Gel Extraction Kit (Axygen Biosciences), and cloned into pMD18-T (TA) (Takara Bio). The plasmid DNA isolated from eight clones of each PCR product was sequenced using the ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

2.10.2. Methylation-specific PCR (MSP)

To analyze the methylation status of the *MT1F* gene, MSP analysis was undertaken using the following primer sequences: sense, 5′-AGGGATGGTTTGTGGTGTGTAGT-3′ and antisense, 5′-CCCAAACACA AAACCCAACA-3′ for unmethylated reactions (312 bp), and sense, 5′-ATGGTTCGCGGTGTGTAGC-3′ and antisense, 5′-CCAAACACGAAACCC AACG-3′ for methylated reactions (307 bp). PCR conditions were as follows: 95 °C for 5 min; 40 cycles of 94 °C for 30 s, 65 °C for 30 s and 68 °C for 60 s; and a final extension of 5 min at 68 °C. PCR products were separated by electrophoresis on 1.5% agarose gels.

2.10.3. LOH analysis

Two fluorescence-labeled primers (Shanghai Biological Technology Ltd, China) for polymorphic microsatellite markers located close to the MT1F locus were used to analyze matched pairs of normal and tumor DNA for LOH analysis. The following primer sequences were used: D16S2878 sense, 5′-CCTATCCATTCCACATCTAAAC-3′ and D 16S2878 antisense 5′-CCTCTCAAAGTATGGGGATTAT-3′ (130 bp) and D16S3008 sense, 5′-AACCACATGACAACTCGCCA-3′ and D16S3008 antisense, 5′-CTTTGGGCGAAGCTAACATC-3′ (106 bp). Amplification was done in a 5-μL volume with 10 ng DNA, 10× standard buffer, 1.5 mmol/L $MgCl_2$, 20 mmol/L deoxynucleotide triphosphates, 0.05 μL Hot-start Taq polymerase (Qiagen, Hilden, Germany) and 0.05 μmol/L of each primer. The cycling conditions were as follows: an initial denaturation at 95 °C for 15 min; 95 °C for 30 s, 63–56 °C for 1.5 min (0.5 °C decreased per cycle), 72 °C for 1 min; 30 cycles each at 94 °C for 30 s, 56 °C for 1.5 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. An ABI 3730 sequencer was used to

analyze PCR products. Through analyzing the fluorescent intensity of each allele and calculating the allele ratio, LOH was determined when the value of allele ratio ≤ 0.67 or ≥ 1.5. A single fragment amplified from normal DNA and those PCR reactions in which fragments were not amplified were considered as non-informative.

$$\text{Allele ratio} = \frac{\text{height of normal allele two}/\text{height of normal allele one}}{\text{height of tumor allele two}/\text{height of tumor allele one}}$$

2.11. Statistical analysis

Data were expressed as means and standard deviations (SDs) for continuous variables or frequencies and percentages for categorical data. The Student's *t* test was used for comparisons of means between two groups; the three-group comparisons were conducted using one way analysis of variance (ANOVA). Multiple comparisons were performed using the Bonferroni procedure with type-I error adjustment for statistically significant values. The associations between categorical variable and outcome were determined using the Fisher's exact test. The level of significance was set at 0.05. Statistical analyses were performed using SAS 9.1 (SAS Institute Inc., Cary, NC) statistical software.

Table 1
Downregulated gene expression in LOH loci.

LOH loci[a]	Gene symbol	Chromosome location	Fold-change[b]	*p*-Value
16q13.1	*MT1F*	chr16:55249335–55250716	−3.7	0.004150
	MT1G	chr16:55258153–55259478	−3.9	0.004150
	MT1X	chr16:55273882–55275462	−3.3	0.037598
	MT2A	chr16:55199996–55200910	−3.3	0.001221
	MT1E	chr16:55216896–55218525	−3.7	0.067627
	MT1H	chr16:55261274–55262541	−3.8	0.067627
	PLLP	chr16:55847509–55876072	−1.7	0.046143
16q24.1	*SPG7*	chr16:88138143–88142270	−1.1	0.080566
	COTL1	chr16:84599204–84651669	−2.7	0.432373
	TCF25	chr16:88467507–88494703	−2.1	0.008057
	HSD17B2	chr16:80626363–80689638	−6.7	0.633789
11p13	*CD59*	chr11:33686714–33714601	−2.2	0.001221
3q28–29	*SST*	chr3:188869393–188870896	−3.3	0.601074
	B3GNT5	chr3:184471286–184473866	−1.1	0.000244
4q34–35	*HPGD*	chr4:175647784–175680550	−4	0.095215
	FBXO8	chr4:175394386–175441386	−1	0.001221
6q25.2–27	*VIP*	chr6:153113734–153122591	−5.9	0.828613
	VIL2	chr6:159106767–159159196	−2.6	0.398926
	TAGAP	chr6:159375566–159376530	−1.6	0.095215
7p21–22	*AGR2*	chr7:16798790–16811222	−1.6	0.000244
	SCIN	chr7:12576536–12659753	−3.7	0.001953
	BCMP11	chr7:16865561–16888137	−4.9	0.010742
7q21–22	*ABCB1*	chr7:86970275–86971055	−1.5	0.010742
	CYP3A4	chr7:99193508–99219649	−1.6	0.149658
	CYP3A7	chr7:99140595–99170757	−1.5	0.432373
	CASD1	chr7:94002610–94024215	−1.6	0.001953
	GNAI1	chr7:79602097–79685286	−2.1	0.246094
	FZD1	chr7:90731718–90736068	−2.2	0.129639
	HGF	chr7:81166256–81237380	−2.9	0.432373
	SRI	chr7:87672365–87687322	−1.7	0.000244
	SAMD9	chr7:92567100–92570485	−4.6	0.696289
	RPIB9	chr7:87299096–87299547	−2.6	0.018555
	LOC253012	chr7:92655834–92656398	−5.7	0.171387
	PDK4	chr7:95050744–95052702	−3	0.001953
9p21–23	*MOBKL2B*	chr9:27319128–27519779	−1.8	0.018555
	CDKN2B	chr9:21992898–21993475	−4.7	0.19458
	LRRC19	chr9:26983587–26995670	−4.4	0.010742

[a] The LOH regions as reported previously [6].
[b] Cancer tissue versus normal tissue.

3. Results

3.1. Genes downregulated in LOH loci

Analysis of downregulated genes mapping to the LOH loci was accomplished by combining the data from the LOH and the gene expression array; 37 genes were mapped to these LOH regions; the fold-changes from array were shown in Table 1.

Because the LOH at 16q chromosome was observed in Chinese colon cancer patients [7], candidate TSGs may exist in this region. Colon cancer tissue was isolated from 40 patients and used for subsequent gene expression analysis. *MT1F*, *MT1G*, *MT1X*, and *MT2A* genes, which are all located on chromosome 16q13.1, were all significantly downregulated in colon cancer specimens (all $p<0.05$; Table 1).

3.2. Reduced transcriptional expression of MT genes in colon cancer

To further confirm whether MT isoforms were differentially expressed in colon cancer as compared to adjacent normal mucosa, their expression levels in 40 colon cancer samples were determined using QRT-PCR. As compared to normal mucosa, MT mRNA expression levels of each isoform were downregulated in the majority (>75%) of the colon cancer samples analyzed (Table 2, Fig. S1). Although MT1F mRNA expression was downregulated, its expression was not associated with clinicopathological features (Table S3). Other MT isoforms were not associated with clinicopathological characteristics too (data not shown).

3.3. Analysis of MT protein expression by TMA immunohistochemistry

To detect MT protein expression in colon cancer, immunohistochemical analysis of TMAs was undertaken using an antibody that recognizes both MT1 and MT2. Of the 203 normal mucosa specimens, 186 (91.6%) had positive MT expression, whereas the absence of MT protein staining was evident in many colon cancer tumors (114/203, 56.2%; Fig. 1). In addition, MT protein expression was decreased in samples with downregulated MT isoform mRNA expression, suggesting that *MT* gene downregulation correlated with decreased protein level. Downregulation of MT protein expression in tumor tissues was confirmed using Western blot analysis (Fig. S2).

Although downregulation of MT1F mRNA expression was not associated with clinicopathological features, reduced MT protein expression was significantly associated with depth of tumor invasion (pT stage), lymph node metastasis (pN stage), and AJCC stage ($p=0.048$, 0.023 and 0.045, respectively). No correlations were found between MT protein expression and age, gender, tumor location, distant metastasis (M stage), histologic grade or vascular invasion (Table S4).

3.4. Exogenous MT1F inhibits RKO cell survival, invasion and adhesion

Because the *MT1F* gene was downregulated in normal-adenoma–carcinoma sequence of colon tissues [18], its mRNA expression was

Table 2
Frequency of MT isoform mRNA downregulation in 40 colon cancer tissues compared with normal mucosa.

MT isoform	Frequencies of transcriptional expression	
	Downregulated	Nondownregulated
MT1E	32 (80.0%)	8 (20.0%)
MT1F	33 (82.5%)	7 (17.5%)
MT1G	32 (80.0%)	8 (20.0%)
MT1H	31 (77.5%)	9 (22.5%)
MT1X	30 (75.0%)	10 (25.0%)
MT2A	31 (77.5%)	9 (22.5%)

Data are represented as counts (percentages).

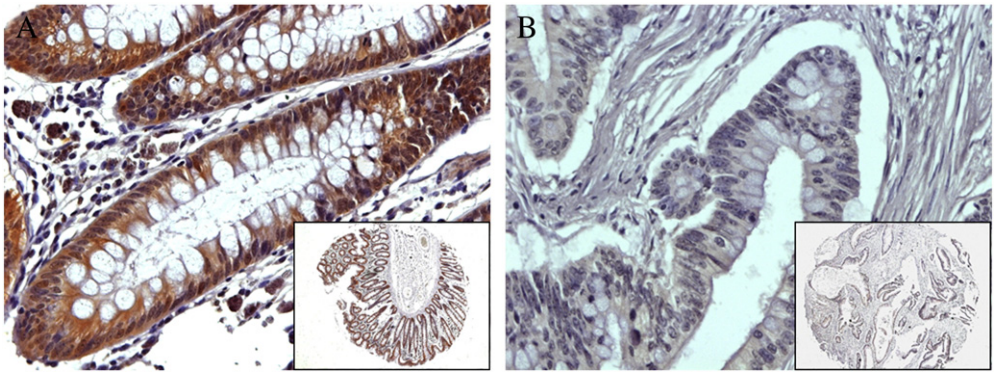

Fig. 1. Immunohistochemical staining of MT protein in normal colon mucosa (A) and cancerous tissues (B) on a TMA (400×). Insets depict the corresponding lower magnification view (50×).

evaluated in colon cancer cell lines. It was almost absent in RKO and LoVo cells as compared to the other cell lines (Fig. S3).

Because MT1F mRNA expression and MT protein expression were undetectable in RKO cells, which have with the capacity to form colonies in agarose gel and tumors in nude mice, the RKO cell line was selected for the subsequent in vitro experiments. In order to examine the influence of MT1F expression on colon cancer cell growth, RKO cells were transfected with an MT1F expression plasmid. Upregulation of MT1F expression was confirmed in transiently and stably transfected cells (Fig. S4A and B, respectively).

Significantly decreased proliferation of MT1F-transfected RKO cells was observed after 2 to 6 days as compared to both control groups (Fig. 2A). In addition, RKO colony formation was significantly decreased upon MT1F expression ($p<0.05$; Fig. 2B). Furthermore, RKO cell apoptosis rate significantly increased from 15.0% and 17.3% among parental and empty vector-transfected cells, respectively to 28.2% in MT1F-transfected cells ($p<0.05$, Fig. 2C). Marked DNA ladders were also observed in MT1F-overexpressing cells, and the changes were not found in cells of other two groups (Fig. 2D). However, no differences in cell cycle distributions were observed (Fig. S5). These results suggest that MT1F inhibits cell growth by inducing RKO cell apoptosis.

The effect of MT1F expression on RKO cell invasion and adhesion was also assessed. Cell migration and invasion were significantly reduced in MT1F-transfected cells as compared to parental and empty vector-transfected cells ($p<0.05$; Fig. 2E). In addition, decreased adhesion to Collagen I and IV was observed in MT1F-expressing RKO cells ($p<0.05$, respectively, Fig. 2F). These data suggest that MT1F could have a potential role in the initial stages of colon cancer progression.

3.5. MT1F inhibits tumor growth in vivo

To evaluate the effect of MT1F on tumorigenesis in vivo, MT1F-cDNA or empty vector-transfected RKO cells were subcutaneously implanted in mice. The growth rate of MT1F-expressing tumors was reduced as compared to control tumors. Specifically, the average tumor size of MT1F-expressing tumors was reduced as compared to empty vector-expressing tumors at day 21 ($1.14\pm0.54\ cm^3$ versus $2.89\pm0.64\ cm^3$, respectively $p<0.05$, Fig. 3A). In addition, the average weight of MT1F-expressing tumors was reduced as compared to controls (1.08 ± 0.38 g versus 2.50 ± 0.45 g, respectively, $p<0.05$, Fig. 3B). These results reveal that MT1F can inhibit RKO tumor growth in vivo.

3.6. High frequency of LOH at MT1F gene locus but not hypermethylation in colon cancer tissues

Promoter hypermethylation, intragenic mutations, or LOH can induce downregulation of certain genes [25]. Therefore, the role of possible CpG island methylation in the loss of MT1F expression in colon cancer cells was assessed. A CG-rich region with 41 CpG sites around the transcriptional start site of MT1F was observed (Fig. 4A). BSP detection (Fig. S6) and MSP detection (Fig. S7) revealed that MT1F gene was methylated in only LoVo and RKO cells, not in other four cell lines. However, there was no MT1F hypermethylation in colon cancerous tissue samples based on MSP detection (Fig. 4B), suggesting that other mechanisms, such as genetic alterations, might involve in silencing of MT1F in colon cancerous tissues.

As MT1F downregulation can occur without gene mutation [26], the LOH status of the *MT1F* gene was assessed in colon cancer tissues using two microsatellite markers, D16S2878 and D16S3008, which were found on chromosome 16q13.1, flanking the MT1F locus. In the 40 pairs of colon cancer specimens, there were 35 and 34 informative cases at each of the D16S2878 and D16S3008 markers, respectively (Table S1). Then, LOH at markers D16S2878 and D16S3008 was observed in 40% (14 of 35) and 47.1% (16 of 34) of the samples, respectively. Five cases exhibited LOH at both markers (Fig. 4C and D, Table S1). Furthermore, 100% (25/25) LOH cases while only 53.8% (7/13) non LOH cases showed downregulation of MT1F. MT1F downregulation was significantly associated with LOH in colon cancer tissues ($p<0.001$, Table 3). However, as with MT1F mRNA expression (Table S3), no significant correlation was found between MT1F LOH status and clinicopathologic features of the patients (Table S5). Taken together, these data indicate that LOH may be the principle mechanism responsible for the inactivation of *MT1F* gene expression in colon cancer tissues.

4. Discussion

In this study, we found that the downregulation of specific *MT* isoforms was observed in colon cancer. The transcriptional expression of MT1F was inactivated by LOH in colon cancer tissues; CpG island hypermethylation was also observed in poorly differentiated, MSI-positive RKO and LoVo colon cancer cell lines but not in tumor tissues.

Because downregulation of *MT* genes has been reported in several tumors, their role as tumor suppressors in colon cancer was explored in this study. Combination analysis of LOH and microarray data has revealed several candidate TSGs [27,28]. Functional studies revealed that *MT* genes might act as tumor suppressors in colon cancer. LOH mediated MT downregulation in colon cancer tissues.

MT1F, *MT1G*, *MT1X*, and *MT2A* gene expression was significantly downregulated in colon cancerous tissues, which is in accordance with previous reports [18,29]. Specifically, expression of the *MT1F* gene gradually decreased from the benign to malignant phenotype [18]. The dysregulation of *MT* genes could possibly relate to the embryological origin of neoplastic lesion. The *MT1F* gene is often downregulated in endodermal hepatic and pharyngeal cancer cells, as well as in mesodermal prostate cancer cells [8]. Although the *MT1F* gene

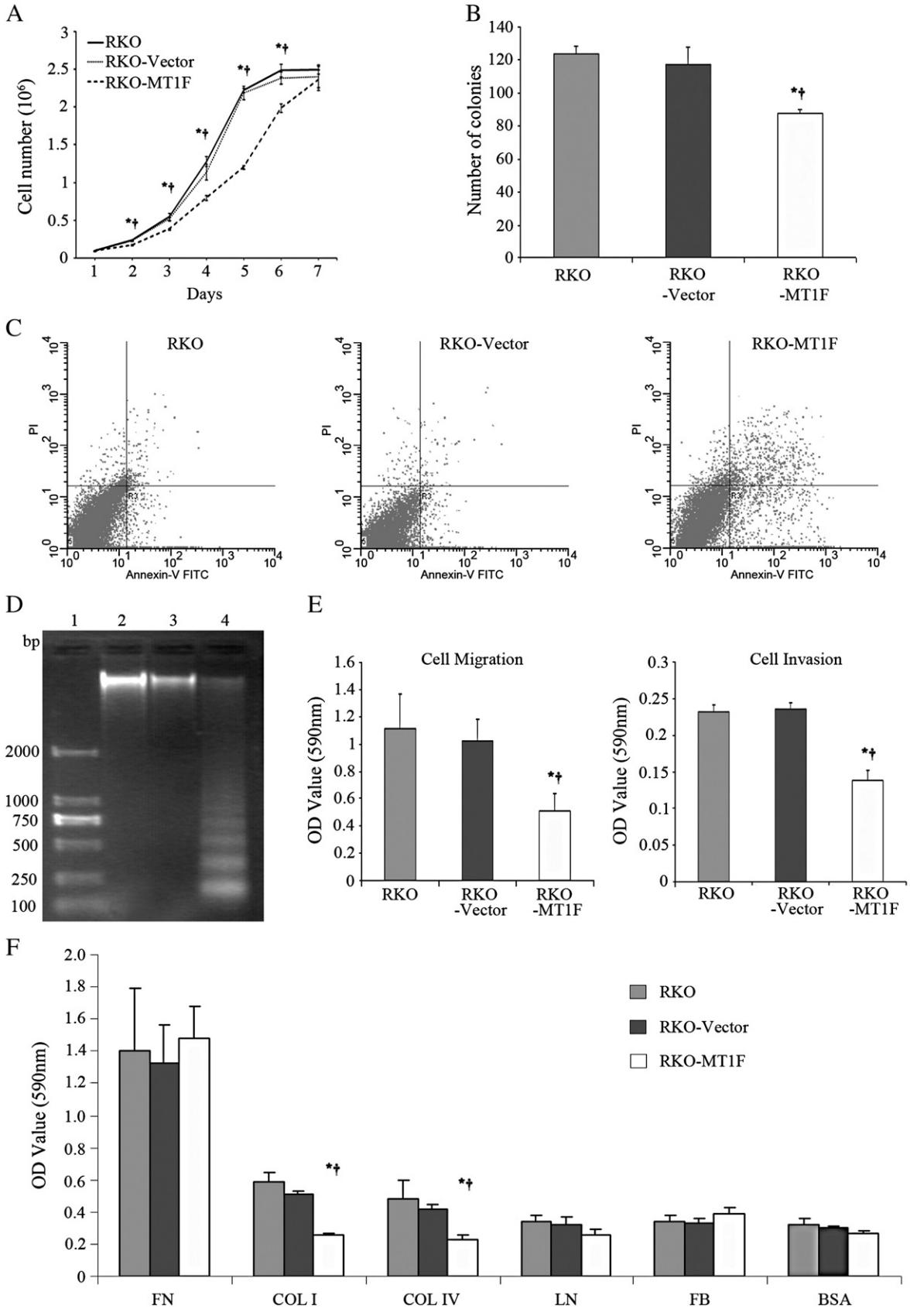

Fig. 2. The effect of MT1F expression on RKO cell proliferation (A), colony formation (B), apoptosis (C,D), migration and invasion (E), and adhesion (F). In panel D, lanes 1 to 4 were DNA marker, RKO cells, empty vector and MT1F transfected cells, respectively. The experiments were repeated in triplicate. Differences among the three groups were analyzed by ANOVA. Multiple comparisons were tested by Bonferroni adjustment with type-I error. Data represent means and SDs. In panel F, RKO cell adhesion to the following extracellular matrix components was determined: FN, Fibronectin; COL I, Collagen I; COL IV, Collagen IV; LN, Laminin; and FB, Fibrinogen. $^{*}p<0.05$, shows the difference between parental and MT1F-transfected RKO cells. $^{\dagger}p<0.05$, shows the difference between vector control and MT1F-transfected cells.

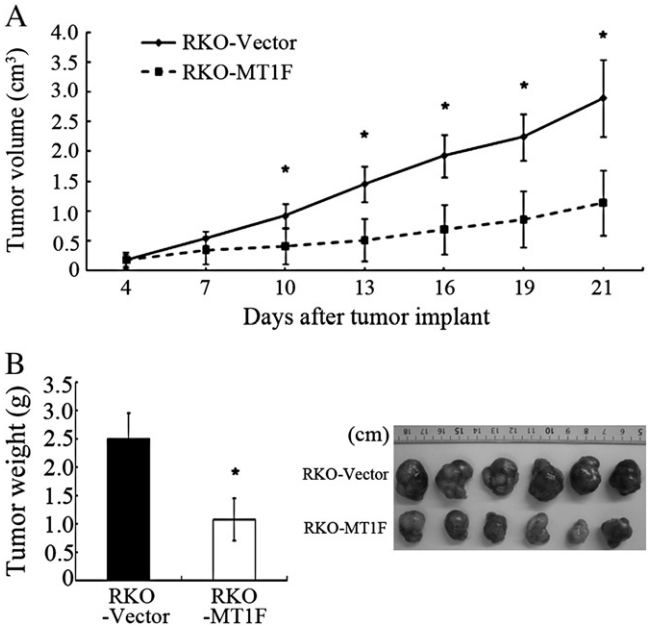

Fig. 3. Inhibition of RKO cell tumorigenicity upon MT1F expression. (A) The tumor volume from six mice was observed in each group every three days from day 4 to day 21. (B) At day 21, the mice were sacrificed, and the tumor weights were measured. Data represent means and SDs. $^{*}p<0.05$, showed a significant difference between MT1F- and vector alone-transfected cells.

was obviously downregulated in endodermal colon cancers, its mRNA levels were not associated with the clinicopathological characteristics of the patients. MT protein was also significantly decreased in tumorous tissues compared with matched normal colonic mucosa, which is in agreement with several previous reports [12–14]. Furthermore,

Table 3
The association between MT1F mRNA expression and LOH in colon cancer tissues.

Downregulation of MT1F	No. of samples[a]		*p*-Value
	LOH	No LOH	
	(no. of cases)	(no. of cases)	
Present	25 (100%)	7 (53.85%)	$<0.001^{\dagger}$
Absent	0 (0%)	6 (46.15%)	

Data were represented as counts (percentages).
$^{\dagger}p<0.05$, showed a significant association between the presence of MT1F downregulation and LOH.
[a] Two samples without effective information of all markers D16S2878 and D16S3008 were not included in analysis.

metallothionein expression was evenly distributed in the cytoplasm and nuclei, which was similar to that previously reported [13,14,16], Similar to other investigations [14,30,31], a significant association between MT protein levels and pT, pN and AJCC stage was observed. In premalignant high-grade dysplasia, reduced MT protein expression is suggestive that it may be an early step in colon carcinogenesis [32,33].

A role for MTs in colon cancer progression has yet to be elucidated. Considering their anti-oxidant, anti-inflammatory and anti-mutagenic properties, MT downregulation may increase susceptibility toxin-induced damage [34]. Indeed, MT-null mice had a higher rate of induced carcinogenesis compared with wild-type mice [35–39]. Inhibition of NF-κB activity by MTs may suppress cancer cell growth and induce apoptosis [40]. MTs also reduce the expression of proteins required for carcinogenesis, including cyclooxygenase-2, nitric oxide synthase, tumor necrosis factor-α, interleukin (IL)-1β and IL-6 expression.8 Furthermore, MT directly interact with p53 and may alter its activity through zinc exchange [41].

In the present study, restoration of *MT1F* gene expression in RKO cells reduced both in vitro proliferation through increased apoptosis

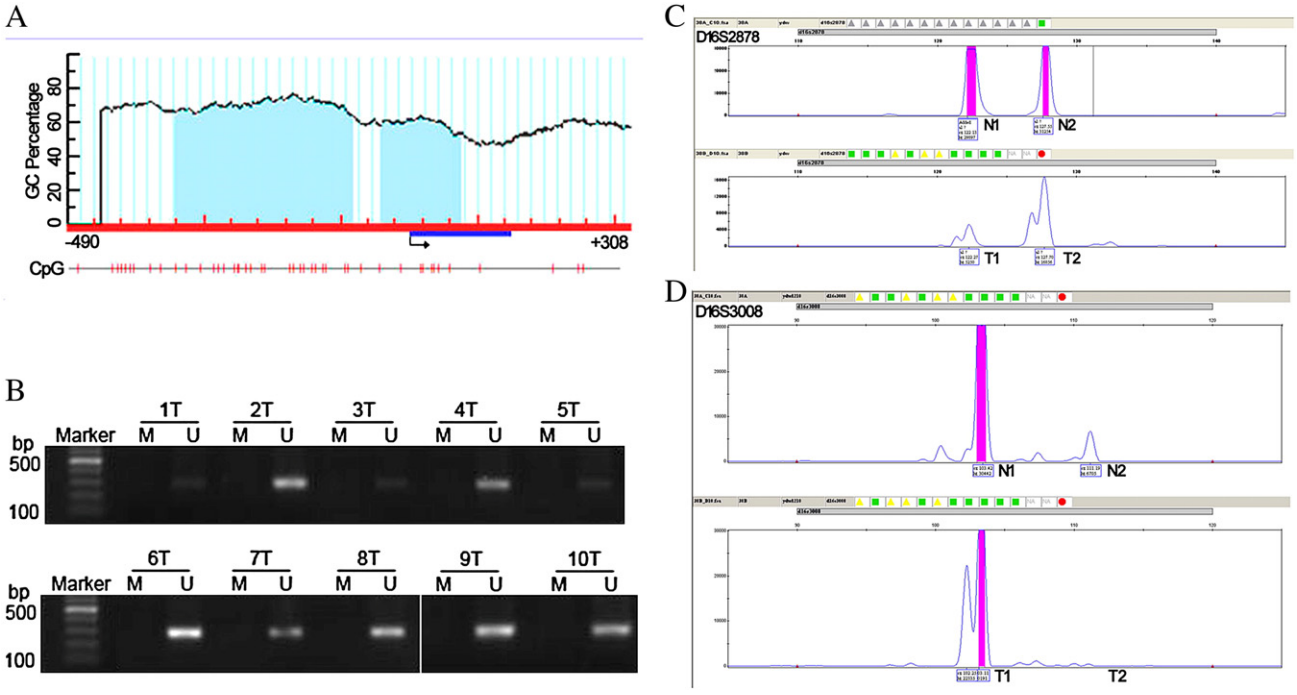

Fig. 4. A representative illustration of LOH at MT1F gene locus but not hypermethylation in colon cancer tissues. (A) The CpG island (%GC = 64.6, Obs./Exp. CpG = 0.652, length = 604 bp) in MT1F gene by using CpG island searcher (http://cpgislands.usc.edu) and software MethPrimer (http://www.urogene.org/methprimer). The arrow indicated the transcriptional start site of MT1F. (B) The representative electrophoresis map of MSP products from colon cancerous tissues for methylation study of MT1F gene. (C) LOH on 2 markers together in the same CC tissues. Marker D16S2878, Allele Ratio = (T1:T2)/(N1:N2) = (5250:16,856)/(29,897:31,254) = 0.33. The value of allele ratio is less than 0.67 and consistent with the LOH criterion. (D) Marker D16S3008, the pattern of a single peak in tumor and two peaks in normal tissue fits well for typical peak of LOH. M: methylated products; U: unmethylated products; T: tumor tissues; N: normal tissues.

and in vivo tumorigenicity, indicating that MT1F might act as a tumor suppressor. This is consistent with Hecht et al. [42] where transfection of the *MT1F* gene into salivary gland cells from submandibular tumors promoted morphologic differentiation in vitro and in vivo, and reduced tumor growth in nude mice. Exogenous *MTIF* gene expression inhibited the growth and tumorigenicity of HepG2 liver cancer cells [26]. MT1F might induce apoptosis by inhibition of NF-κB activity; however, the molecular mechanisms governing the effects of MT1F expression on colon cancer cell growth and tumorigenicity requires further exploration.

MT expression was significantly reduced in the metastatic colon cancer lesions and inversely correlated with CD44 expression [14]. Colon cancer cells with MT downregulation also gave rise to the hepatic metastasis, suggesting that MTs may modulate the metastatic process [15,43]. In line with those reports, restoration of MT1F expression inhibited RKO cell migration, invasion and adhesion in vitro. Further studies are necessary to determine the mechanism by which MT expression influences metastasis in vivo.

The mechanisms leading to MT mRNA downregulation in colon cancer were explored in the present study; hypermethylation was associated with MT1F downregulation only in RKO and LoVo cell lines, which is consistent with previous reports [44–48]. For example, CpG hypermethylation of MT1E was present in endometrial cancer and melanoma [44,45]; MT1G hypermethylation was reported in hepatoblastoma, breast and lung cancers [46–48]. However, in our study, no evidence of methylation was found in colon cancer tissues. It remains possible that some parenchymal cells of a different subtype or miscellaneous mesenchymocytes were hypermethylated, but not others. Moreover, it is possible that only one allele was methylated, thereby affecting the results. Differences in *MT1H*, *MT1X*, and *MT2A* gene expression have been observed between microsatellite instability (MSI) and microsatellite stability (MSS) colon cancer [49]. RKO and LoVo cell lines are poorly differentiated and have MSI whereas SW620 and HT29 are MSS and well differentiated [50]. Our results indicated that MT1F mRNA expression in RKO and LoVo cell lines was significantly down-regulated with hypermethylation at its promoter region in comparison to CW-2 and HT29 cell lines. Thus, downregulation and hypermethylation of the MT1F gene might be associated with MSI in colon cancer. These results are different from those reported for MT2A in which no differences were observed in several colon cancer cell lines [51]. Further studies are necessary to determine if the methylation status of MT1F is related to the differentiation status of colon cancer cells.

Loss of MT expression may also be attributed to LOH [8]. Chromosomal LOH was associated with MT1F mRNA downregulation in colon cancer tissues in the present study. Because *MT1F* gene downregulation often occurs absent gene mutations in hepatocellular carcinoma [26], the *MTIF* gene may be deleted through LOH; however, this has to be confirmed in further studies. Inactivation of a TSG typically takes place in two steps: a small deletion or germline mutation inherited from parents and a large deletion of part of a chromosome in somatic cells that involves a particular gene [52]. Detection of LOH can help to understand the molecular mechanisms of tumor development and provide important information about disease diagnosis and prognosis [53]. *MT1F* gene LOH was more frequently observed in tumors with a diameter less than 5 cm with a well differentiated grade and histotype. Although the differences were not statistically significant, this trend suggested that MT1F LOH might be an early event in colon cancer progression. As MT1F gene LOH was not present in a minority of colon cancerous tissues, further analysis of the downregulation of *MT* genes in primary colon carcinomas is required.

The present study has limitations that warrant discussion. MT1F was the only isoform analyzed. The effects of downregulation of the other MT isoforms require further investigation. In addition, the mechanism by which MT1F expression decreased tumor size was not analyzed. Although increased RKO cell apoptosis was observed upon MT1F expression in vitro, it remains to be elucidated whether the same effect is observed in vivo. Finally, the effects of MT1F expression were only analyzed in RKO cells. Determining the effects of MT1F knockdown in cells that express high levels of MT1F expression, including CW-2 and HCT116 cells, as well as normal cells is necessary to support that the *MT1F* gene is a tumor suppressor; this will be determined in further studies.

Taken together, the downregulation of specific *MT* isoforms was observed in colon cancer. Loss of heterozygosity is main reason for MT1F mRNA downregulation in colon cancer. A putative tumor suppressor role for the MT1F isoform in colon cancer was observed. However, further explorations are required to fully elucidate the molecular mechanisms governing *MT* gene dysregulation and its role in colon cancer progression.

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Disclosure/conflict of interest

The authors have no conflicts of interest.

Statement of translational relevance

This article presented down-regulated expression of *Metallothionein 1F* in colon tumor tissues and human colon cancer cell lines, the mechanism of down-regulation of *Metallothionein 1F* by loss of heterozygosity in majority of colon tumor tissues and colon cancer cell lines. The author found that over-expression of *Metallothionein 1F* gene could inhibit tumor growth in a nude mouse model. Metallothionein 1F protein expression was negatively associated with status of tumor invasion, lymph node metastasis and AJCC stage. In colon cancer cell lines, over-expression of *Metallothionein 1F* could induce apoptosis and inhibit migration, invasion and adhesion. It is strongly indicated that *Metallothionein 1F* is a tumor suppressor gene during colon tumorigenesis. Restoration or over-expression of *Metallothionein 1F* gene in cancer cells might represent a novel approach in clinic for colon cancer gene therapy. The expression status of *Metallothionein 1F* gene might be used in clinic as a predictive biomarker for colon tumor development of prognosis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbadis.2012.02.021.

References

[1] A. Jemal, R. Siegel, E. Ward, Y. Hao, J. Xu, M.J. Thun, Cancer statistics, 2009, CA. Cancer J. Clin. 59 (2009) 225–249.
[2] S. Zhang, Y. Cui, Z. Weng, X. Gong, M. Chen, B. Zhong, Changes on the disease pattern of primary colorectal cancers in Southern China: a retrospective study of 20 years, Int. J. Colorectal Dis. 24 (2009) 943–949.
[3] W.M. Grady, J.M. Carethers, Genomic and epigenetic instability in colorectal cancer pathogenesis, Gastroenterology 135 (2008) 1079–1099.
[4] M.S. Pino, D.C. Chung, The chromosomal instability pathway in colon cancer, Gastroenterology 138 (2010) 2059–2072.

[5] L. Luo, G.Q. Shen, K.A. Stiffler, Q.K. Wang, T.G. Pretlow, T.P. Pretlow, Loss of heterozygosity in human aberrant crypt foci (ACF), a putative precursor of colon cancer, Carcinogenesis 27 (2006) 1153–1159.
[6] Z. Peng, F. Zhang, C. Zhou, Y. Ling, S. Bai, W. Liu, et al., Genome-wide search for loss of heterozygosity in Chinese patients with sporadic colorectal cancer, Int. J. Gastrointest. Cancer 34 (2003) 39–48.
[7] X.Y. Xiao, X.Y. Zhou, G. Yan, M.H. Sun, X. Du, Chromosomal alteration in Chinese sporadic colorectal carcinomas detected by comparative genomic hybridization, Diagn. Mol. Pathol. 16 (2007) 96–103.
[8] T. Eckschlager, V. Adam, J. Hrabeta, K. Figova, R. Kizek, Metallothioneins and cancer, Curr. Protein Pept. Sci. 10 (2009) 360–375.
[9] M.G. Cherian, A. Jayasurya, B.H. Bay, Metallothioneins in human tumors and potential roles in carcinogenesis, Mutat. Res. 533 (2003) 201–209.
[10] A.T. Miles, G.M. Hawksworth, J.H. Beattie, V. Rodilla, Induction, regulation, degradation, and biological significance of mammalian metallothioneins, Crit. Rev. Biochem. Mol. Biol. 35 (2000) 35–70.
[11] F. Haq, M. Mahoney, J. Koropatnick, Signaling events for metallothionein induction, Mutat. Res. 533 (2003) 211–226.
[12] A.M. Janssen, W. van Duijn, F.J. Kubben, G. Griffioen, C.B. Lamers, J.H. van Krieken, et al., Prognostic significance of metallothionein in human gastrointestinal cancer, Clin. Cancer Res. 8 (2002) 1889–1896.
[13] A.M. Janssen, W. van Duijn, M.M. Oostendorp-Van De Ruit, L. Kruidenier, C.B. Bosman, G. Griffioen, et al., Metallothionein in human gastrointestinal cancer, J. Pathol. 192 (2000) 293–300.
[14] E.E. Ioachim, A.C. Goussia, N.J. Agnantis, M. Machera, E.V. Tsianos, A.M. Kappas, Prognostic evaluation of metallothionein expression in human colorectal neoplasms, J. Clin. Pathol. 52 (1999) 876–879.
[15] Y. Hishikawa, H. Kohno, S. Ueda, T. Kimoto, D.K. Dhar, H. Kubota, et al., Expression of metallothionein in colorectal cancers and synchronous liver metastases, Oncology 61 (2001) 162–167.
[16] P. Dziegiel, J. Forgacz, E. Suder, P. Surowiak, J. Kornafel, M. Zabel, Prognostic significance of metallothionein expression in correlation with Ki-67 expression in adenocarcinomas of large intestine, Histol. Histopathol. 18 (2003) 401–407.
[17] I. Sutoh, H. Kohno, Y. Nakashima, Y. Hishikawa, H. Tabara, M. Tachibana, et al., Concurrent expressions of metallothionein, glutathione S-transferase-pi, and P-glycoprotein in colorectal cancers, Dis. Colon Rectum 43 (2000) 221–232.
[18] S. Lee, S. Bang, K. Song, I. Lee, Differential expression in normal-adenoma–carcinoma sequence suggests complex molecular carcinogenesis in colon, Oncol. Rep. 16 (2006) 747–754.
[19] A.J. Wilson, A.C. Chueh, L. Tögel, G.A. Corner, N. Ahmed, S. Goel, et al., Apoptotic sensitivity of colon cancer cells to histone deacetylase inhibitors is mediated by an Sp1/Sp3-activated transcriptional program involving immediate-early gene induction, Cancer Res. 70 (2010) 609–620.
[20] J. Fan, Z. Peng, C. Zhou, G. Qiu, H. Tang, Y. Sun, et al., Gene-expression profiling in Chinese patients with colon cancer by coupling experimental and bioinformatic genomewide gene-expression analyses: identification and validation of IFITM3 as a biomarker of early colon carcinogenesis, Cancer 113 (2008) 266–275.
[21] J.B. O'Connell, M.A. Maggard, C.Y. Ko, Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging, J. Natl. Cancer Inst. 96 (2004) 1420–1425.
[22] D.W. Yan, D.W. Li, Y.X. Yang, J. Xia, X.L. Wang, C.Z. Zhou, et al., Ubiquitin D is correlated with colon cancer progression and predicts recurrence for stage II–III disease after curative surgery, Br. J. Cancer 103 (2010) 961–969.
[23] S. Mididoddi, J.P. McGuirt, M.A. Sens, J.H. Todd, D.A. Sens, Isoform-specific expression of metallothionein mRNA in the developing and adult human kidney, Toxicol. Lett. 85 (1996) 17–27.
[24] S.K. Tai, O.J. Tan, V.T. Chow, R. Jin, J.L. Jones, et al., Differential expression of metallothionein 1 and 2 isoforms in breast cancer lines with different invasive potential: identification of a novel nonsilent metallothionein-1H mutant variant, Am. J. Pathol. 163 (2003) 2009–2019.
[25] S.R. Payne, C.J. Kemp, Tumor suppressor genetics, Carcinogenesis 26 (2003) 2031–2045.
[26] D.D. Lu, Y.C. Chen, X.R. Zhang, X.R. Cao, H.Y. Jiang, L. Yao, The relationship between metallothionein-1F (MT1F) gene and hepatocellular carcinoma, Yale J. Biol. Med. 76 (2003) 55–62.
[27] Y. Yi, J. Mirosevich, Y. Shyr, R. Matusik, A.L. George Jr., Coupled analysis of gene expression and chromosomal location, Genomics 85 (2005) 401–412.
[28] L. Hawthorn, J. Luce, L. Stein, J. Rothschild, Integration of transcript expression, copy number and LOH analysis of infiltrating ductal carcinoma of the breast, BMC Cancer 10 (2010) 460.
[29] K. Okuno, M. Yasutomi, N. Nishimura, T. Arakawa, M. Shiomi, J. Hida, et al., Gene expression analysis in colorectal cancer using practical DNA array filter, Dis. Colon Rectum 44 (2001) 295–299.
[30] P. Dziegiel, M. Dumańska, J. Forgacz, A. Wojna, M. Zabel, Intensity of apoptosis as related to the expression of metallothionein (MT), caspase-3 (cas-3) and Ki-67 antigen and the survival time of patients with primary colorectal adenocarcinomas, Rocz. Akad. Med. Bialymst 49 (2004) 5–7.
[31] K.J. Schmitz, C.I. Müller, H. Reis, H. Alakus, G. Winde, H.A. Baba, et al., Combined analysis of hypoxia-inducible factor 1 alpha and metallothionein indicates an aggressive subtype of colorectal carcinoma, Int. J. Colorectal Dis. 24 (2009) 1287–1296.
[32] M. Bruewer, K.W. Schmid, C.F. Krieglstein, N. Senninger, G. Schuermann, Metallothionein: early marker in the carcinogenesis of ulcerative colitis-associated colorectal carcinoma, World J. Surg. 26 (2002) 726–731.
[33] D. Laukens, H. Peeters, A. Waeytens, S. Bogaert, B.V. Cruyssen, D. Elewaut, et al., Reduced metallothionein expression in colonic Crohn's disease: evidence for it new disease-modifying gene, Gastroenterology 134 (2008) A457-A457.
[34] C.D. Tran, S. Sundar, G.S. Howarth, Dietary zinc supplementation and methotrexate-induced small intestinal mucositis in metallothionein-knockout and wild-type mice, Cancer Biol. Ther. 8 (2009) 1662–1667.
[35] B. Zhang, M. Satoh, N. Nishimura, J.S. Suzuki, H. Sone, Y. Aoki, et al., Metallothionein deficiency promotes mouse skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene, Cancer Res. 58 (1998) 4044–4046.
[36] M.P. Waalkes, J. Liu, R.A. Goyer, B.A. Diwan, Metallothionein-I/II double knockout mice are hypersensitive to lead-induced kidney carcinogenesis: role of inclusion body formation, Cancer Res. 64 (2004) 7766–7772.
[37] J.S. Suzuki, N. Nishimura, B. Zhang, Y. Nakatsuru, S. Kobayashi, M. Satoh, et al., Metallothionein deficiency enhances skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoylphorbol-13-acetate in metallothionein-null mice, Carcinogenesis 24 (2003) 1123–1132.
[38] K. Shibuya, N. Nishimura, J.S. Suzuki, C. Tohyama, A. Naganuma, M. Satoh, Role of metallothionein as a protective factor against radiation carcinogenesis, J. Toxicol. Sci. 33 (2008) 651–655.
[39] M. Takaishi, A. Shimada, J.S. Suzuki, M. Satoh, H. Nagase, Involvement of metallothionein (MT) as a biological protective factor against carcinogenesis induced by benzo[a]pyrene (B[a]P), J. Toxicol. Sci. 35 (2010) 225–230.
[40] A. Sakurai, S. Hara, N. Okano, Y. Kondo, J. Inoue, N. Imura, Regulatory role of metallothionein in NF-kappaB activation, FEBS Lett. 455 (1999) 55–58.
[41] E.A. Ostrakhovitch, P.E. Olsson, S. Jiang, M.G. Cherian, Interaction of metallothionein with tumor suppressor p53 protein, FEBS Lett. 580 (2006) 1235–1238.
[42] D. Hecht, D. Jung, V.V. Prabhu, P.J. Munson, M.P. Hoffman, H.K. Kleinman, Metallothionein promotes laminin-1-induced acinar differentiation in vitro and reduces tumor growth in vivo, Cancer Res. 62 (2002) 5370–5374.
[43] U. Stenram, B. Ohlsson, K.G. Tranberg, Immunohistochemical expression of metallothionein in resected hepatic primary tumors and colorectal carcinoma metastases, APMIS 107 (1999) 420–424.
[44] W.J. Faller, M. Rafferty, S. Hegarty, G. Gremel, D. Ryan, M.F. Fraga, et al., Metallothionein 1E is methylated in malignant melanoma and increases sensitivity to cisplatin-induced apoptosis, Melanoma Res. 20 (2010) 392–400.
[45] K.Y. Tse, V.W. Liu, D.W. Chan, P.M. Chiu, K.F. Tam, K.K. Chan, et al., Epigenetic alteration of the metallothionein 1E gene in human endometrial carcinomas, Tumour Biol. 30 (2009) 93–99.
[46] L.H. Sakamoto, B. D.E. Camargo, M. Cajaiba, F.A. Soares, A.L. Vettore, MT1G hypermethylation: a potential prognostic marker for hepatoblastoma, Pediatr. Res. 67 (2010) 387–393.
[47] S.Y. Park, H.J. Kwon, H.E. Lee, H.S. Ryu, S.W. Kim, J.H. Kim, et al., Promoter CpG island hypermethylation during breast cancer progression, Virchows Arch. 458 (2011) 73–84.
[48] Q. Lin, J. Geng, K. Ma, J. Yu, J. Sun, Z. Shen, et al., RASSF1A, APC, ESR1, ABCB1 and HOXC9, but not p16INK4A, DAPK1, PTEN and MT1G genes were frequently methylated in the stage I non-small cell lung cancer in China, J. Cancer Res. Clin. Oncol. 135 (2009) 1675–1684.
[49] C.P. Giacomini, S.Y. Leung, X. Chen, S.T. Yuen, Y.H. Kim, E. Bair, et al., A gene expression signature of genetic instability in colon cancer, Cancer Res. 65 (2005) 9200–9205.
[50] G. Deng, A. Chen, J. Hong, H.S. Chae, Y.S. Kim, Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression, Cancer Res. 59 (1999) 2029–2033.
[51] S. John, T. Briatka, E. Rudolf, Diverse sensitivity of cells representing various stages of colon carcinogenesis to increased extracellular zinc: implications for zinc chemoprevention, Oncol Rep. 25 (2011) 769–780.
[52] A.G. Knudson Jr., Mutation and cancer: statistical study of retinoblastoma, Proc. Natl. Acad. Sci. U. S. A. 68 (1971) 820–823.
[53] A. Velasco, J. Pallares, M. Santacana, A. Yeramian, X. Dolcet, N. Eritja, et al., Loss of heterozygosity in endometrial carcinoma, Int. J. Gynecol. Pathol. 27 (2008) 305–317.